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(54) **Method for preparing proteins using transformed lactic acid bacteria.**

(57) Method for preparing proteins by means of host organisms, in particular lactic acid bacteria, which contain recombinant DNA plasmides with a high replicon activity and a high promoter activity for the protein-coding gene concerned; examples of proteins to be prepared are phospho-β-galactosidase and chymosin or a precursor thereof.

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Method for preparing proteins by means of host organisms, in particular lactic acid bacteria, which contain recombinant DNA plasmids with a high replicon activity and a high promoter activity for the protein-coding gene concerned, the host organisms provided with such recombinant DNA plasmids, the recombinant DNA plasmids themselves, the DNA fragments coding replicon activity and promoter activity, as w ll as the proteins obtained.

The invention relates to the application of recombinant DNA technology with the aim of producing in an efficient manner polypeptides in bacteria, in particular in lactic acid bacteria, in the bacterium B. subtilis and in the bacterium E. coli. More particularly the invention relates to the provision of plasmid vectors which replicate in both Gram-negative and Gram-positive bacteria, including lactic acid bacteria, as host organisms, and which are suitable for cloning and bringing genes to expression in said bacteria, in particular a phospho-β-galactosidase gene. The invention relates very particularly to the provision of DNA fragments or plasmid vectors with a replicon, a promoter, a ribosome binding site and a gene which advantageously originates from lactic acid bacteria and which functions in lactic acid bacteria, in B. subtilis and in E. coli, and also to a method of preparation for these. The invention also relates to a method for cloning genes in lactic acid bacteria. Finally the invention embraces the host organisms which are provided with one or mor plasmid vectors according to the invention.

The development and the application of recombinant DNA technology in genetic model systems such as the Gram-negative E. coli and the Gram-positive B. subtilis has shown that bacteria are capable of producing foreign (heterologous) polypeptides in an efficient manner and, in addition, can be stimulated to an increased or reduced production of species-intrinsic (homologous) polypeptides. The basic elements for the application of said recombinant DNA technology are:

1. a vector, generally a plasmid, into which homologous or heterologous genes can be introduced in order to achieve stable replication and high expression of said genes. A vector contains one or more DNA fragments which ensure a stable replication (replicon) and, in order to simplify genetic selection, is often endowed with easily selectable genes such as antibiotic-resistance genes;

2. a DNA fragment containing a gene which codes for the required property, which may be a species-intrinsic (homologous) or a foreign (heterologous) property, which DNA fragment is provided with a suitable controlling DNA sequence;

3. one or more DNA fragments containing controlling DNA sequences which are necessary for expression in the host organism, such as a promoter (a DNA sequence which ensures efficient transcription of the gene to be brought to expression) and a ribosome binding site (a DNA sequence which initiates th efficient translation of the transcript formed);

4. a suitable host bacterium in which the vector containing the required gene having the required controlling DNA sequence can be transformed and can be stably maintained, and which possesses the cellular systems to bring the gene concerned to expression.

The application of recombinant DNA technology has led to the production of polypeptides which possess enzymatic activity and catalyse biological conversions such as α-amylase and glucose-isomerase, or which find pharmaceutical application, such as insulin and interferon. Auxiliary substances for the foodstuffs industry such as chymosin, β-galactosidase and β-glucanase, can apparently be produced by the use of recombinant DNA technology.

For the application of recombinant DNA technology in bacteria, mainly E. coli, and to a lesser extent B. subtilis have hitherto been used as host and production organisms. The most important reason for this is that said bacteria are genetically best characterized and are used as model systems for Gram-negative (E. coli) and Gram-positive (B. subtilis)bacteria. All the initial discoveries and inventions of recombinant DNA technology were made using E. coli as host. However, E. coli and B. subtilis are not the most suitabl bacteria for the commercial production of polypeptides for use in the foodstuffs industry: viz., E. coli may produce toxic and pyrogenic compounds and is therefore not permissible in foodstuffs, while B. subtilis is a very active producer of protease, as a result of which the yield of most of the polypeptides produced is very considerably reduced. For nutrient fermentations, which generally proceed in an anaerobic medium, said bacteria are either totally unsuitable (B. subtilis: obligate aerobe) or less suitable (E. coli). Moreover, the application of both types of bacteria is considerably limited in the foodstuffs industry because the taste resulting from the presence of said bacteria is found to be especially unpleasant. This is a considerable drawback especially for application in dairy products: contaminations with B. subtilis or E. coli bring about unacceptable changes in taste.

Lactic acid bacteria have already been used for thousands of years in the preparation of fermented foodstuffs and consumed in large quantities; these bacteria can safely be used. In addition, lactic acid bacteria make an important contribution to the consistency and the taste of said products, and large-scale anaerobic fermentations by means of these microaerophilic bacteria are possible in a simple manner. Lactic acid bacteria are therefore pre-eminently suitable as Gram-positive host and production organisms in the application of recombinant DNA technology in the foodstuffs industry.

The mesophilic lactic acid bacteria belonging to the genera Streptococcus lactis and S. cremoris are used in the preparation of a great variety of dairy products such as cheese, curd cheese, butter and buttermilk. It has emerged that said lactic acid bacteria generally have a plasmid complement which consists of various plasmids varying in number from one to more than six, with molecular weights from approximately 10^6 to over 100×10^6 Although most of these plasmids have no known function (so-called cryptic plasmids) it has been shown that in a number of cases important functions such as lactose and citrate metabolism, proteinase production, bacteriocine production and resistance and bacteriophage resistance mechanisms are wholly or partly coded by plasmid DNA.

By analogy with host-vector systems for the genetic model bacteria E. coli and B. subtilis, endogenous plasmids can be used for the construction of vectors for lactic acid bacteria. An example of this is the recently described vector pGK12 based on the replicon of the S. cremoris plasmid pWV01. Said vector replicates in S. lactis, B. subtilis and E. coli. Said vector is, however, less suitable for the application of recombinant DNA technology in lactic acid bacteria because the copy number of said vector in the lactic acid bacterium S. lactis is very low (approximately four copies per cell; Appl. Environ. Microbiol. 48 (1984) 726-731). Another example is the vector pGB301 which is made up of an S. sanguis replicon and which appears to replicate in the lactic acid bacterium S. lactis (Appl. Environ. Microbiol. 48 (1984) 252-259). The drawback of said vector is also the relatively low copy number and the large size (9.8 kb; Plasmid 4 (1938) 130-138).

It is known from the results which have hitherto been obtained using recombinant DNA technology that the level of gene expression of cloned genes in bacterial systems is strongly related to the copy number of said genes and therefore to the copy number of the vectors used for the cloning. Vectors which have a small size and which replicate with a high copy number in lactic acid bacteria were hitherto unknown.

This invention relates to a vector with a small size which exhibits a high copy number in lactic acid bacteria and to a method which leads to the construction of such a vector. Said method comprises a so-called replicon screening strategy which makes use of the model organism B. subtilis as an intermediate host for the initial selection of DNA fragments with replicon activity. This use is based on the fact that B. subtilis can be transformed with a much higher frequency than lactic acid bacteria (Mol. Gen. Genet. 168 - (1979) 111-115; Appl. Environ. Microbiol. 43 (1982) 1213-1215) and on the likeness which obviously exists between said Gram-positive organisms in relation to replicon activity since the lactic acid bacterium plasmid pWV01 originating from S. cremoris appears to replicate in B. subtilis (Lactic Acid Bacteria in Foods, published by Neth. Soc. Micro-biol. (1983) p. 33).

An important advantage of said method is, moreover, that each constructed vector will replicate in B. subtilis, which increases the gene manipulation possibilities.

An essential component of the replicon screening strategy described here is the development of a plasmid which replicates not in B. subtilis or lactic acid bacteria but in E. coli and, in addition, has Gram-positive antibiotic-resistance genes which are situated on a suitable and small restriction DNA fragment and which are expressed in E. coli; B. subtilis and, as expected, in lactic acid bacteria as well (replicon screening vector). The ligation of said restriction fragment, which contains the antibiotic-resistance genes, with restriction fragments of plasmids originating from and replicating in lactic acid bacteria followed by transformation of B. subtilis may result in antibiotic-resistant transformants. The plasmid DNA isolated from said transformants is then transformed into plasmid-free or plasmid-containing lactic acid bacteria, and antibiotic-resistant lactic acid bacteria are selected. The structure and the copy number of the plasmids in the transformed lactic acid bacteria found is then determined by means of standard techniques. By using the method described above with the replicon screen vector pNZ10 screening, a number of new vectors has been developed which are capable of transforming a lactic acid bacterium. A special case of this is the vector pNZ12 having a size of 4.1 kb, which is provided with genes which bring about resistance to the antibiotics chloramphenicol and kanamycin and which replicates and is maintained with a high copy number (approximately 100 copies per cell) in lactic acid bacteria. In addition to replication in lactic acid bacteria and in B. subtilis, the vector pNZ12 is also capable of transforming the Gram-negative model bacterium E. coli. Moreover, the unique SalI and BglII sites in the vector are of importance in cloning experiments in said hosts. At th se sites it is possible to insert in DNA fragments which, after transforming the recombinant plasmids into lactic acid bacteria, continue to be maintained in the required lactic acid bacterium with a high

copy number. A special case occurs on inserting DNA into the BglII site which is situated in the kanamycin-resistance gene. As a result of this, this marker is inactivated, which makes simple genetic selection possible (insertion inactivation; W.M. de Vos, academic thesis of the State University of Groningen, 1983, Bariet, Ruinen).

Direct gene cloning by means of shotgun experiments in lactic acid bacteria is impeded to a considerable degree by the absence of an efficient transformation system. Although the transformation frequencies which were initially reached in S. lactis (Appl. Environ. Microbiol. 43 (1982) 1212-1215) have been increased by some technical modifications by a factor of ten to approximately 10^3 transformants per μg.(Appl. Environ. Microbiol. 48 (1984) 252-259), an optimized transformation system as described for E. coli or B. subtilis (10^7 transformants per μg) has not been achieved for lactic acid bacteria. Gene clonings in lactic acid bacteria will therefore generally have to be carried out by using intermediate hosts. The v ctor pNZ12 described here replicates both in E. coli and B. subtilis and in lactic acid bacteria and is therefore extremely suitable for using the genetically readily accessible hosts E. coli and B. subtilis in gene clonings aimed at lactic acid bacteria. The use described above of intermediate hosts in gene cloning in lactic acid bacteria, with the use of E. coli and B. subtilis by means of the vector pNZ12 as a preferred embodiment, is a component of the present invention. In this manner, by making use of the unique SalI site in the vector pNZ12, a DNA fragment which originated from S. lactis and which contained the phospho-β-galactosidas gene was cloned in E. coli . Said recombinant plasmid pNZ32 was then transformed in both B. subtilis and a lactic acid bacterium. In all the said hosts the phospho-β-galactosidase gene is expressed. Said expression can be determined in a very simple manner via an enzymatic reaction, as a result of which a new possibility is presented for measuring gene expression in E. coli and B. subtilis, but in particular in lactic acid bacteria.

To bring a gene to expression in a lactic acid bacterium said gene always has to have upstream two controlling DNA elements which are functional in lactic acid bacteria: a promoter and a ribosome binding site. Genes which already have these two DNA elements (such as, in general, homologous genes) can therefore be expressed, but the level of gene expression can generally be increased if a second strong r promoter is placed upstream. Genes which are only provided with a ribosome binding site can only be brought to expression by placing a promoter upstream. Genes which have neither of the two controlling DNA elements (such as, generally, heterologous genes) can only be brought to expression if they are provided with said DNA elements. For this purpose, the gene to be brought to expression is coupled to a gene which does contain a promoter and a ribosome binding site. When the reading frames of the two genes are contiguous, a fusion protein is produced. In some cases said fusion protein may possess the required biological activity. In other cases it is sometimes possible to liberate the required protein from such a fusion protein.

The nature of the promoter and of the ribosome binding site to a large extent determines the expression of the gene situated downstream. DNA fragments on which a promoter and a ribosome binding place are located and which originate from, and function in, lactic acid bacteria, were hitherto unknown, as a result of which progress in the application of recombinant DNA technology in said bacteria was impeded to a large extent. The invention now offers a method for isolating DNA fragments specific to lactic acid bacteria, on which fragments said two controlling DNA sequences are located. The method developed for this purpose is based on the use of a so-called promoter screening vector and of E. coli and B. subtilis as intermediary hosts. Such vectors have been used successfully for cloning promoter fragments in E. coli and B. subtilis and generally consist of a vector containing an antibiotic-resistance gene which has been stripped of its own promoter and is not therefore (or is very poorly) expressed. By introducing usable unique restriction sites just upstream of the gene decapitated in this manner, the applicability of a promoter screening v ctor is considerably increased. Isolation of controlling DNA elements can then be carried out by inserting DNA fragments into said unique restriction sites followed by transforming the intermediary host B. subtilis or E. coli and selecting transformants in which the decapitated antibiotic-resistance gene is again brought to expression (antibiotic-resistant transformants). The DNA fragments which are used for welding in may be derived from chromosomal DNA, plasmid DNA and bacteriophage DNA of lactic acid bacteria. It is known that powerful promoters primarily occur on bacteriophage DNA.

Application of the method described above to the promoter screening vector pNZ220, which is derived from the vector pNZ12 described above and therefore replicates in B. subtilis, in E. coli and in a lactic acid bacterium, results in the isolation of the expression plasmid pNZ221 which contains a DNA fragment originating from a lactic acid bacteriophage which has strong promoter activity in said hosts.

On closer analysis it emerged that said DNA fragment probably contains two promoters which are located immediately in sequence and which have the same orientation. Moreover, said promoters appeared to be followed at a short distance by a ribosome binding site, a start codon (ATG), a so-called open reading frame and, finally, a number of usable restriction sites. As those who are skilled in the art will realise, said DNA fragment specific to lactic acid bacteria and the information mentioned here can be used extremely well for bringing homologous and heterologous genes to expression in lactic acid bacteria.

The vectors and derived plasmids described here are able to replicate both in lactic acid bacteria and in B. subtilis and E. coli. As a result of this, all the gene manipulation techniques which the development of recombinant DNA technology has realized and has still to realise in these model organisms and are still to be realized can be applied to said vectors and derived plasmids.

Until now in lactic acid bacteria only the transformation of S. lactis strains which have been deprived of their natural plasmid DNA content (Appl. Environ. Microbiol. 48 (1984) 252-259; 726-731) in the laboratory has been described. But natural S. lactis strains containing plasmids also appear to be transformable with the vector described here. In addition, it is possible to transfer plasmids from one lactic acid bacterium to another in an efficient manner by means of conjugation. This applies also to the vectors described here. In this manner it is possible to realize various usable plasmid combinations in lactic acid bacteria without being limited by the low transformation frequency which has hitherto been achieved with said organisms. Therefore, the fact that until now S. lactis has been the only transformable lactic acid bacterium does not rule out the possibility of other lactic acid bacteria being used as a host for the vector and derived plasmids described here. In particular S. cremoris will be a very suitable host for the application of the expression-controlling DNA elements present in the expression vector pNZ221 and originating from an S. cremoris phage.

Application of the vector described here, the expression vector derived therefrom and the cloning system for lactic acid bacteria may lead to the development of lactic acid bacteria which produce polypeptides in an efficient and stable manner. Those who are skilled in the art will realize that use of said lactic acid bacteria offers very great advantages in the foodstuffs industry. Thus, increased and stabilized production of enzymes which are a component of the proteolytic system of a lactic acid bacterium (such as proteinases and peptidases) will result in an acceleration of the development of the required taste of a diary product such as, for example, cheese. Moreover, the bacteriophage resistance of a lactic acid bacterium can be enhanced by increasing and stabilizing the expression of genes which prevent bacteriophage adsorption or by cloning and expressing restriction modification systems. Similarly, lactic acid bacteria can be developed which have the ability to produce chymosin or other milk-curdling enzymes.

Example I

Construction of the replicon screening vector pNZ10.

To isolate and identify DNA fragments which have replicon activity in lactic acid bacteria, a replicon screening vector termed pNZ10 was constructed. E. coli plasmid pAT153 (Nature 283 (1980) 216-218) and B. subtilis plasmid pGL112 (Mol. Gen. Genet. 190 (1983) 56-62) were used as the starting point for this purpose. A physical map of both plasmids is shown in Figure 1. The plasmid pGL112 consists of three small TaqI fragments which originate from pUB110 (J. Bacteriol. 134 (1978) 318-329) and which contain the replication signals of said plasmid (Plasmid 6 (1981) 67-77; J. Bacteriol. 154 (1983) 1184-1194) and a Tag I fragment having a size of 2.5 kb. This last named fragment contains the TaqI-HpaII fragment with a size of 1.3 kb from pUB110 which contains the kanamycin (Km)-resistance gene (J. Bacteriol. 160 (1984) 413-420) and the HpaII-TaqI fragment having a size of 1.2 kb from pC194 which contains the chloramphenicol (Cm)-resitance gene (J. Bacteriol. 150 (1982) 815-825). These two antibiotic-resistance markers are functional not only in Gram-positive bacteria such as S. aureus and B. subtilis, but also in E. coli (Proc. Natl. Acad. Sci. 75 (1978) 1433-1436; Mol. Gen. Genet. 183 (1981) 220-226).

To construct pNZ10, 5 µg of pGL112 DNA were isolated as described (Mol. Gen. Genet. 181 (1981) 424-433) from B. subtilis strain IG33, completely digested with units of the restriction enzyme TaqI (Boehringer, Mannheim) in 50 µl of a solution containing 10 mM tris.HCl (pH 7.5), 10 mM $MgCl_2$ and 10 mM 2-mercaptoethanol at 65°C for 2 hours. In addition, 5 µg of pAT153 DNA, isolated as described (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1982) from E. coli strain HB101, were linearized with 20 units of the restriction enzyme ClaI (Boehringer, Mannheim) in 50 µl of a solution containing 10-mM tris.HCl (pH 8.0) and 10 mM $MgCl_2$ at 37°C for 2 hours. Th restriction enzyme fragm nts were then separated by electrophoresis in a 0.7% agarose gel (Appl. Environ. Microbiol. 43 (1982) 1272-1277) and the pAT153 DNA

linearized with ClaI and also the TaqI fragment of pGL112 with a size of 2.5 kb were isolated as described - (Anal. Bioch m. 66 (1975) 213-220) from the gel. The isolated DNA fragments were mixed, precipitated with ethanol (Biochim. Biophys. Acta 607 (1980) 1-9) and then dissolved in 17 µl of TE buffer (10 mM tris.HCl - (pH 7.5); 1 mM EDTA), heated for 10 minutes at 68°C, and cooled to 4°C. Then 2 µl of 10-fold concentrated ligase buffer (500 mM tris.HCl (pH 7.5); 60 mM $MgCl_2$; 10 mM ATP; 50 mM dithiothreitol) and 1 unit of T4 DNA ligase (Boehringer, Mannheim) were added and the mixture was finally incubated at 14°C overnight. The restriction enzyme ClaI recognizes the sequence CTCGAG and generates protruding CG 5' ends: the restriction enzyme TaqI recognizes the sequence TCGA and also generates protruding CG 5' ends, so that ClaI and TaqI fragments can be ligated in a simple manner ("sticky end" ligation). Moreover Cla -Taq fusions can always be cut again by the restriction enzyme TaqI but they do not need to be recognized by the restriction enzyme ClaI. Half of the ligation mixture was used to transform E. coli strain BHB2600 as described (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor). Transformants resistant to ampicillin (50 µg/ml) and chloramphenicol (10 µg/ml) were isolated with high frequency (2.1×10^3 per µg of DNA). From some of these transformants the plasmid DNA was isolated on a micro scale - (Nucl. Acids. Res. 7 (1979) 1513-1523) and analysed by means of, inter alia, TaqI digestions. One of the transformants contained a plasmid called pNZ10 with a structure as shown in Figure 1. Said plasmid pNZ10 replicates in E. coli by means of the pAT153 replicon and appears to bring about resistance to kanamycin - (10 µg/ml) in said host. The pAT153 replicon is not, however, functional in B. subtilis nor in lactic acid bacteria such as S. lactis, as a result of which pNZ10 does not replicate in said bacteria and is therefore extremely suitable as a replicon screening vector. In addition, the pAT153 section of pNZ10 contains a large number of TaqI-recognition sites so that TaqI digestion of pNZ10 DNA leaves intact only the 2.5 kb fragment on which the antibiotic-resistance genes are located as shown in Figure 1.

Example II

Use of the replicon screening vector pNZ10 in the construction of the cloning vector pNZ12.

The usability of the replicon screening, vector can be checked in experiments in which pNZ10 DNA digested with the restriction enzyme TaqI, is ligated with restriction fragments which contain protruding CG 5' ends (as formed ih digestion with the restriction enzymes ClaI, TaqI, AsuII, AcyI, MaeII, MspI, NarI and also their isoschizomers, and in some cases AccI; see Gene 33 (1985) 1-102) originating from, preferably, plasmid DNA of lactic acid bacteria. The ligation mixture can be used to transform B. subtilis protoplasts and to select Cm-and/or Km-resistant transformants. These transformants then contain recombinant plasmids which consist at least of the 2.5 kb pNZ10 Taq I fragment on which the antibiotic-resistance genes are located and also DNA fragments with replicon activity in B. subtilis. Since such fragments are not present in pNZ10 (see Example I) selection occurs for DNA fragments with a replicon activity originating from lactic acid bacteria. It is of course possible to isolate DNA fragments containing replicon activity and originating from other bacteria by replacing the lactic acid bacterium DNA in said procedure by DNA from the desired bacterium.

One of the vectors which has been constructed in the manner described above is the cloning vector pNZ12. For said construction plasmid DNA was completely isolated from the lactic acid bacterium strain S. lactis NCD0712 (J. Bacteriol. 154 (1983) 1-9) by a modification of the alkaline extraction method (Nucl. Acids Res. 7 (1979) 1513-1523; Third European Congress on Biotechnology, published by Verlag Chemie, III, 201-206). For this purpose the lactic acid bacteria were cultivated in 1 litre of glucose-M17 medium - (Appl. Microbiol. 29 (1975) 807-813) to a E_{∞} of approximately 1.0 and harvested by centrifuging (10 minutes at 6,000 rpm; GSA rotor Sorvall) at 4°C, washed with sucrose buffer (25% sucrose; 20 mM tris.HCl (pH 8.0); 100 mM NaCl; 5 mM EDTA) and then suspended again in 75 ml sucrose buffer containing 500 µg of lysozyme (55700 u/ml; Sigma) per ml. This mixture was heated at 37°C until a clarification indicating complete "lysis" was observed (generally after 10 to 15 min. of incubation at 37°C) in micro-scale test experiments in which 100 µl of mixture was mixed with 200 µl of hydroxide solution (0.2N NaOH; 1% sodium dodecyl sulphate). At that instant 150 ml of freshly prepared hydroxide solution were added to 75 ml of mixture and the entire amount was kept on ice for 10 minutes after carefully mixing. Then 115 ml of ice-cold 3 M potassium acetate solution (pH 4.5 containing glacial acetic acid) were added; after mixing, the whole amount was kept on ice again for 10 min. After centrifuging (20 min.) at 4°C, the supernatant was filtered through cheesecloth in order to remove pellet particles which had been stirred up. The nucleic acids were precipitated from said supernatant with ethanol or isopropanol and subjected to CsCl-ethidium bromide equilibrium centrifugation as described (Appl. Environ. Microbiol. 43 (1982) 1272-1277) in order to

isolate the plasmid DNA. Five µg of the S. lactis NCD0712 plasmid DNA thus isolated were again dispersed with the restriction enzyme TaqI and mixed with 2 µg of pNZ10 DNA also digested with the restriction enzyme Taq I . The restriction enzyme was removed by a phenol extraction followed by a chloroform extraction, and the DNA was precipitated with ethanol and then ligated in a final volume of 100 µl as described. Half of this ligation mixture was used to transform B. subtilis strain IG33 protoplasts as described (Mol. Gen. Genet. 168 (1979) 111-115; Mol. Gen. Genet. 182 (1981) 39-43) and Cm^R transformants were selected. One of said transformants contained a small (4.1 kb) plasmid which also produced Km^R in B. subtilis as well as Cm^R. The physical map of said plasmid pNZ12 is also shown in Figure 1 and reveals that there are various unique restriction enzyme recognition sites in pN212, inter alia for the restriction enzymes BglII and ApaI (both located in the Km^R gene) and SalI. By means of hybridization experiments (Molecular Cloning; A Laboratory Manual, Cold Spring Harbor, 1982) it was possible to establish that pNZ12, in addition to the 2.5 kb TaqI fragment of pNZ10, contains two smaller TaqI fragments (1079 and 599 bp) which originate from the smallest S. lactis NCD0712 plasmid, pSH71 (J. Bacteriol. 154 (1983) 1-9). The full nucleotide sequence of said plasmid with a size of 2,060 bp has been determined by standard sequencing methods (Proc. Natl. Acad. Sci. 74 (1977) 5463-5467; Methods in Enzymology 101 (1983) 20-78; ibid. 65 - (1980) 499-560) and is shown in Figure 2 together with a physical map of pSH71. The nucleotide sequence of the DNA with replicon activity present in pNZ12, which embraces the pSH71 DNA from position 2-1680, can be derived from this.

Those skilled in the art will realize that it is also possible to construct plasmids consisting of pNZ12 DNA, extended with one or more of the other five TaqI fragments of pSH71 which replicate in the same hosts as pNZ12. Moreover it has emerged that so-called mini-plasmids consisting of the 2.5 kb TaqI fragment of pNZ12 and the 599 bp Taq I fragment of pSH71 are also capable of replicating in B. subtilis and E. coli if the 2-1680 sequence of pSH71 is present on another replicon.

Example III

Determination of the host range and of the copy number of plasmid pNZ12.

In order to investigate the hosts in which pNZ12 can be used as a vector, pNZ12 DNA was transform d into representatives of both Gram-positive and Gram-negative bacteria of which it was expected that the antibiotic-resistance genes would come to expression therein. For this purpose E. coli strain MC1061 (J. Bacteriol. 143 (1983) 971-980) was transformed with 0.5 µg of pNZ12 DNA which resulted in approximat ly 10^4 transformants resistant to 10 µg of Cm/ml. Each of the twenty transformants investigated more closely for plasmid content contained a plasmid with the physical structure of pNZ12. Moreover, it emerged that pNZ12 also produced resistance to Km in E. coli (10 µg/ml). Protoplasts of S. aureus strain RN451 (J. Gen. Microbiol. 33 (1963) 121-136) were transformed as described (Mol. Gen. Genet. 168 (1979) 111-115; Proc. Natl. Acad. Sci. 79 (1982) 4108-4112) with 1 µg of pNZ12 DNA resulting in approximately 10^3 transformants resistant to 10 µg Cm/ml and 50 µg Km/ml. Some of these transformants were used to isolate plasmid DNA by the alkaline extraction method described above, starting from 1 ml of cell suspension and with an extra addition of 30 µg of lysostaphin per ml (220 µ/mg; Sigma) during the lysis step. In all the twelve transformants investigated there was plasmid DNA which had the physical structure of pNZ12.

S. lactis strain MG1363 (J. Bacteriol. 154 (1983) 1-9) was transformed by means of the protoplast-transformation system as follows: 50 ml of cells cultivated in glucose-M17 broth at 30°C to an E_{∞} of approximately 0.8 were harvested by centrifuging (10 minutes, 6.000 rpm; SS34 rotor Sorvall) at 20°C, and resuspended in 50 ml of GSM17 (M17 broth containing 0.5 M sucrose and l% glucose) which contained 40 mM ammonium acetate buffer (pH 7.0) and 1 mM magnesium acetate (FEMS Microbiol. Letters 9 (1980) 99-102) and also 1-4 mg of lysozyme per ml (55700 u/mg; Sigma) and then incubated for 2 hours at 37°C. The protoplasts were collected by centrifuging (10 minutes, 12,000 rpm; SS34 rotor Sorvall) at 20°C, washed with 20 ml of SMMC buffer (SMM buffer (Mol. Gen. Genet. 168 (1979) 111-115) with 10 mM $CaCl_2$) and resuspended in 5 ml of SMMC. The transformation of the washed protoplasts was carried out by adding to 100 µl of the suspension 10 µl of pNZ12 DNA which had been previously mixed with an equal volume of twice concentrated SMMC containing 1 µg of pNZ12 DNA, followed by addition of 300 µl of 40% polyethylene glycol 6000 in SMMC. After incubating for 5 minutes at 20°C, 1 ml of GSM17 was added, the protoplasts were collected by centrifuging (1 minute, 12,000 rpm in an Eppendorf 5414 S centrifuge) and dissolved in 1 ml of GSM17. After an expression period of 1 hour at 30°C various dilutions of the protoplasts prepared in GSM17 and solidified in 3 ml of GSM17 with 0.7% agar were plated out on GSM17 agar plates containing 1.5% agar and 4 µg Cm/ml.

After three days of incubation at 30°C, approxiately 10^3 Cm ^{R} transformants were found per µg of pNZ12 DNA. Some of these transformants were used to isolate plasmid DNA by the alkaline extraction method described above, starting from 1 ml of cell suspension. In all the transformants investigated there was plasmid DNA which had the physical structure of pNZ12.

The copy number of pNZ12 in the hosts B. subtilis, E. coli and S. lactis was determined by a hybridization method in which all the DNA was liberated from a known quantity of logarithmically growing cells and transferred to Gene Screen Plus (New England Nuclear) filters as described (Nucl. Acids Res. 7 - (1979) 1541-1550). Said filters were then hybridized with ^{32}P-labelled pNZ12 DNA (J. Mol. Biol. 113 (1977) 237-251), washed, dried and autoradiographed (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor 1982). The quantity of radioactivity (a measure of the quantity of pNZ12 DNA in the samples) was then determined and compared with the quantity of radioactivity found in a hybridization experiment carried out in parallel, in which known quantities of pure pNZ12 DNA were placed on the filters. Allowing for the number of bacteria in the initial culture, it was possible to derive the copy number of pNZ12 per bacterium cell from these data. From the results listed in Table 1 it emerges that pNZ12 has a copy number in E. coli and in S. lactis which is high and comparable with that in the cloning vector pAT153 (100 -150 copies per cell) much used in E. coli. Moreover, it emerged from the determination of the plasmid DNA yield that pNZ12 also has such a high copy number in S. aureus. In B. subtilis, on the other hand, the copy number of pNZ12 is a factor of five lower.

TABLE 1

Host		Copy number of pNZ12 per cell
E. coli	MC1061	98
B. subtilis	IG33	19
S. lactis	MG1363	110

Example IV

Cloning of the S. lactis phospho-β-D-galactosidase gene in the vector plasmid pNZ12 and expression in E. coli, B. subtilis and S. lactis .

To check the usability of the cloning vector pNZ12 described above, the phospho-β-D-galactosidase (P-β-Gal) gene of S. lactis was cloned in S. lactis, use being made of intermediary hosts in which pNZ12 can replicate. The P-β-Gal gene was chosen because the gene product can be determined spectrophotometrically in a simple manner by means of the chromogenic substrate o-nitrophenyl-β-D-galactopyranoside-6-phosphate (Agric. Biol. Chem. 43 (1979) 2389-2390) and is therefore very usable as a model gene for measuring gene expression. The starting point was the plasmid pSM76, which consists of pAT153 in which the ClaI-SalI fragment is replaced by a 3.5 kb ClaI-XhoI fragment from the S. lactis NCD0712 plasmid pLP712 which contains the P-β-Gal gene (Genetic Engineering of Microorganisms Important for Agro-Food Industries, CEC, Marseille (1984) 34-35). Because the SalI-XhoI fusion is not recognized by SalI or XhoI, the P-β-Gal gene was liberated from pSM76 by digestion with ClaI and Na I. It had been found previously that NaeI does not cut the S. lactis section of pSM76 while a Nae I recognition site is located 120 bp from the ClaI-XhoI fusion in the pAT153 section (Cold Spring Harbor Symp. Quant. Biol. 43 (1978) 77-85). Because, furthermore, the restriction enzyme NaeI generates DNA fragments with so-called blunt ends, a blunt-end ligation was chosen in order to insert the DNA fragment which contains the P-β-Gal gene into pNZ12; see Figure 3, in which fSN indicates the SalI-NaeI fusion. For this purpose 5 µg of pSM76 DNA wer digested with ClaI as described, the lin ar DNA was precipitated with ethanol and tak n up in 50 µl of polym rase buff r (25 mM tris.HCl, pH 7.8; 10 mM $MgCl_2$; 10 mM dithiothreitol; 0.1 mM dATP; 0.1 mM dGTP; 0.1 mM dCTP; 0.1 mM TTP). The ClaI recognition sit was then filled in by adding 5 units of DNA polymerase I large (Kl now) fragment (Boehring r, Mannheim) and incubating for 20 minutes at 20°C. Th r action was stopped by incubating for 10 minutes at 65°C and th DNA was precipitated with thanol, then tak n up in 50 µl of NaeI buffer (10 mM tris.HCl, pH 7.5; 10 mM $MgCl_2$; 50 mM NaCl; 10 mM

2-mercaptoethanol) and digested with 20 units of the restriction enzyme NaeI (Boehringer, Mannheim) for 2 hours at 37°C. The 3.9 kb fragment which contains th P-β-Gal gene was then separated from the pAT153 fragments by means of agarose-gel electrophoresis, isolated, precipitated with thanol, and taken up in 20 µl of TE buffer (see Example I). The vector pNZ12 was linearized and provided with blunt ends as follows: 5 µg of pNZ12 were digested with 20 units of the restriction enzyme SalI (Boehringer, Mannheim) in 50 µl of SalI buffer (50 mM tris.HCl, pH 7.5; 100 mM NaCl; 10 mM $MgCl_2$; 10 mM 2-mercaptoethanol) for 2 hours at 37°C. The linearized pNZ12 DNA was then precipitated with ethanol and taken up in 50 µl of polymerase buffer. The SalI ends were filled in with DNA polymerase I large (Klenow) fragment as described, and the DNA was separated by means of agarose-gel electrophoresis, isolated, precipitated with ethanol and taken up in 20 µl of TE buffer. Ten µl of the filled-in ClaI-NaeI fragment described above which contains the P-β-Gal gene and 10 µl of the vector pNZ12, linearized with SalI and also filled in, were combined and, after adding 2.5 µl of 10-fold concentrated ligase buffer, were ligated with 2.5 units of T4 DNA polymerase (see Example I) for 20 hours at 15°C. Ten µl of said ligation mixture was used to transform E. coli strain MC1061 to Cm^RKm^R. Transformants containing the P-β-Gal gene were identified by means of colony hybridization (Proc. Natl. Acad. Sci. 72 (1975) 3961-3966) with ^{32}P-labelled pSM76 DNA. One of the positive clones contained a plasmid pNZ32 with the structure shown in Figure 3. After treatment with toluene, cells which contained pNZ32 exhibited P-β-Gal activity measured as explained earlier, which indicates that the intact S. lactis P-β-Gal gene was cloned in pNZ12. In order to demonstrate the usability of pNZ12 as a vector for P-β-Gal-gene when cloning in B. subtilis and in S. lactis, pNZ32 DNA was isolated from E. coli strain MC1061 and used to transform protoplasts of B. subtilis and of S. lactis as described in Example II. In B. subtilis strain IG33 approximately 10^5 Cm^RKm^R transformants were found per µg; from some of these plasmid DNA was isolated, which was identical to pNZ32 as regards physical organization.

In S. lactis strain MG1363 approximately 10^3 Cm^R tranformants were found per µg; here too the physical organization of the plasmid DNA of some of these transformants proved to be identical to that of pNZ32. In both Gram-positive bacteria, as well as in E. coli , the P-β-Gal gene is expressed (see Table 2). This indicates that pNZ12 is a usable cloning vector for said hosts. Moreover, this means that the P-β-Gal gene is a usable model gene both for E. coli and B. subtilis and for lactic acid bacteria.

TABLE 2

P-β-Gal activity (nmol/min/mg of protein)

		without pNZ32	with pNZ32
E. coli	MC1061	1	186
B. subtilis	IG33	0.1	10
S. lactis	MG1363	1	58

Example V

Construction of the promoter screening vector pNZ220.

The B. subtilis plasmid pPL603 is a so-called promoter screening vector which can be used to isolate DNA fragments with promoter activity (J. Bacteriol. 148 (1981) 1162-1165). For this purpose, in addition to the replication functions and the Km^R gene of pUB110 (see Example I), said plasmid also contains the structural gene which codes for the enzyme chloramphenicol acetyl-transferase (CAT) originating from B. pumilis which is deprived of the CAT promoter active in vegetative cells. Immediately upstream of the CAT gene there is located an EcoRI-PstI fragment with a size of 203 pb, which can be replaced in a simple mann r by the EcoRI-PstI polylinker sequence as has been described for the construction of pPL703 for which the 21 pb polylinker from M13mp7 was used (J. Bacteriol. 155 (1983) 1399-1406). As a result of this not only extra, unique r striction enzyme recognition sites are created immediately upstream of the structural CAT gen but, in addition, said manipulation completely deprives the CAT gene of promoter sequenc s which are functional in B. subtilis (J. Bacteriol. 158 (1984) 784-790). In an analogous manner the

pPL603 EcoRI-PstI fragment is replaced in the plasmid pNZ22 used here (Figure 4) by the 28 bp Eco-Pst polylinker from M13mp8 (Gene 19 (1984) 269-276), as a result of which unique restriction enzyme recognition sites for SmaI, BamHI and Sal I are added. In Figure 4 the recognition sites for the restriction enzymes EcoRI, XmaI, BamHI, SalI, PstI, HindIII, TaqI, and MboI are indicated respectively by the letters E, X, B, S, P, H, T, and M. MboI is an isoschizomer of Sau3A, and XmaI of SmaI. The location of the antibiotic resistance genes is indicated, the CAT gene of B. pumilis promotorless being indicated by a broken line. The emboldened lines in pNZ12 and pNZ22 indicate those sections which are combined in pNZ220.

The usability of a promoter screening vector such as pNZ22 can be considerably increased by replacing the pUB110 replication functions by those from pNZ12 (see Examples II and III), as a result of which the host range is expanded. Because of the favourable location of the Sau3A restriction enzyme recognition sites and the presence of the same Km^R gene (the kanamycin nucleotidyltransferase KNT gene from pUB110, J. Bacteriol. 160 (1984) 413-420) in pNZ22 and pNZ12, this can be done in a simple manner by replacing the 2.2 kb BamHI-BglII fragment of pNZ22 by the 3.5 kb Sau3A fragment of pNZ12 (see Fig. 4). Because, moreover, the Sau3A, BglII and BamHI restriction fragments contain the same 5' GATC protruding ends, sticky end ligation is possible. In addition, in said construction, the BamHI recognition site is reformed again by the sequence GATCC.... in the small Sau3A-SalI fragment of pNZ12 (originating from pC194: J. Bacteriol. 150 (1982) 815-825). An example of such a plasmid is the promoter screening vector pNZ220. The starting point for the construction thereof was 5 µg of pNZ22 DNA which were digested with 20 units of the restriction enzymes BamHI and BglII (Boehringer, Mannheim) in 50 µl of SalI buffer - (Example IV) for 2 hours at 37°C. The restriction fragments were separated by means of agarose-gel electro phoresis, and the BamHI-BglII fragment with a size of 2.2 kb was isolated and concentrated as described above and dissolved in 20 µl of TE buffer. Five µg of pNZ12 DNA were digested in 50 µl of SalI buffer with 10 units of the restriction enzyme Sau3A (Boehringer, Mannheim) for 2 hours at 37°C, and the 3.5 kb Sau3A fragment was isolated and concentrated as described after separation, and dissolved in 20 µl of TE buffer. Ten µl of each of the two fragments, the BamHI-BglII fragment of pNZ22 and the Sau3A fragment of pNZ12, were mixed and ligated in a volume of 50 µl by means of T4 DNA ligase as described. This ligation mixture was used to transform E. coli strain MC1061 as described, and transformants which are resistant to 6 µg Km/ml were selected with a frequency of approximately 10^2 per µg of DNA. In one of these transformants there was a plasmid, pNZ220, with the structure shown in Figure 4. Said plasmid contains the replication function of pNZ12 which makes replicatopm in B.subtilis, E.coli, S.aureus and lactic acid bacteria possible.

By now linearizing pNZ22 or pNZ220 with one of the restriction enzymes which recognize recognition sites situated upstream of the CAT gene and ligating DNA fragments of various origins, but preferably originating from lactic acid bacteria, to the linearized plasmid DNA, recombinant plasmids can be constructed which can be transformed into B.subtilis, and in the case of pNZ220 additionally into E.coli, S.aureus and lactic acid bacteria. Transformants which are resistant to Cm can then have DNA fragments with promoter activity, the level of Cm resistance being a measure of the strength of the promoter.

Example VI

Construction of the expression vector pNZ221.

The usability of the promoter screening vectors pNZ22 and pNZ220 for isolating DNA fragments with promoter activity was checked in the construction of the expression vector pNZ221, consisting of pNZ220 containing a Sau3A fragment on which a strongly lactic-acid-specific promoter is located. The virulent S.cremoris bacteriophage 0 SK11G (FEMS Microbiol. Letters 23 (1984) 175-178) was chosen as a sourc of DNA with promoter activity. It is known that bacteriophages generally have very powerful promoters (Gene 15 (1981) 81-93; Proc.Natl.Acad.Sci. 78 (1981) 4936-4940; J.Mol.Biol. 153 (1981) 527-544). ØSK11G bacteriophage was multiplied on S.cremoris strain SK112 and purified as described (FEMS Microbiol.Letters 23 (1984) 175-178). From approximately 10^{13} ØSK11G particles approximately 500 µg of ØSK11G DNA were isolated as described for bacteriophage lambda (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor 1982). Th ØSK11G DNA is a linear double-stranded DNA with a size of approximately 50 kb and contains cohesive ends. The ØSK11G DNA was dissolved in TE buffer to a concentration of 500 µg/ml. Ten µl of said ØSK11G DNA were completely digested with the restriction enzyme Sau 3A under conditions described in Example V. The restriction enzyme was removed by a phenol extraction and a chloroform extraction, and the DNA fragments were precipitated with ethanol and dissolved in 20 µl of TE buffer. Then 5 µg of pNZ22 DNA were linearized with the restriction enzyme Bam HI as described above (Example V).

deproteinized, precipitated, and dissolved in 50 µl of TE buffer. Ten µl of this linear pNZ22 DNA were mixed with 20 µl of the 0SK11G-Sau3A fragments described above, and the DNA mixture was ligated with T4 DNA ligase in a volume of 50 µl. Half of this ligation mixture was used to transform protoplasts of B.subtilis strain IG33, and transformants which were resistant to 10 µg Cm/ml were selected; approximately 10^3 of such transformants were found. One of the said transformants proved resistant to approximately 75 µg Cm/ml, and the plasmid DNA in this transformant was investigated more closely. Said plasmid, pNZ250, contained a Sau3A fragment with a size of 495 bp, originating from 0SK11G as it was possible to establish by means of hybridization experiments, in the BamHI recognition site of pNZ22. The nucleotide sequence of said 495 bp fragment having promoter activity has been determined by means of standard sequencing methods (see Example II) and is shown in Figure 5. In said figure two promoter sequences are underlined and an RBS is shown by a dotted line; in addition, an open reading frame is shown which starts with an ATG initiation codon and with translation into amino acids. From the DNA sequence around the Sau 3A recognition sites it may be deduced that said sites in pNZ250 are also recognized by the restriction enzyme BamHI. As a result of this it is possible to liberate the 495 bp promoter fragment in a simple manner from pNZ250 by digestion with the restriction enzyme BamHI. Use was made of this in subcloning the 495 bp promoter fragment in pNZ220. For this purpose 5 µg of pNZ250 DNA were digested with the restriction enzyme BamHI; the promoter fragment was isolated after polyacrylamide gel electrophoresis (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor 1982), concentrated and dissolved in 10 µl of TE buffer. Then 5 µg of pNZ220 DNA were linearized with the restriction enzyme BamHI, deproteinized, concentrated and taken up in 50 µl of TE buffer. Ten µl of each of the fragments were ligated by means of T4 DNA ligase in a volume of 50 µl, and the ligation mixture was used to transform B.subtilis 1G33 protoplasts and Cm^Rtransformants were analysed for their plasmid content. In each of the transformants investigated there was a plasmid with a structure like that of pNZ221 (Figure 6). Because the replication functions of pNZ12 - (see Examples II and III) are present in pNZ221, this expression plasmid can be used not only in B.subtilis but also in E.coli , S.aureus and lactic acid bacteria.

The location and the orientation of the DNA sequences with promoter activity in the 495 bp BamHI fragment of pNZ221 were investigated by means of subcloning experiments, use being made, inter alia, of the asymmetric HaeIII recognition site (see Figure 5, position 163). Only the righthand HaeIII-BamHI fragment proved to have promoter activity in both B.subtilis and E.coli. In the DNA sequence of this fragment section (see Figure 5) two regions appear to occur which exhibit homology with the promoter consenus DNA sequence which has been drawn up for E.coli and B.subtilis promoters (Mol.Gen.Genet. 186 (1982) 339-346). These two inferred S.cremoris 0SK11G promoters are followed by the sequence AGAAAG-GACG (see Figure 5), which exhibits strong complementarity with the 3′ ends of the 16S rRNA of the Gram-positive bacteria B.subtilis, S.aureus and S.lactis (J.Biol.Chem. 256 (1981) 11283-11292; J.Gen.Microbiol. 131 (1985) 543-551), and can therefore function as so-called ribosome binding site. The fact that there is located at eleven bp from said RBS a start codon (ATG) which is followed by a so-called open reading frame which comprises 61 codons up to the Bam HI recognition site, suggests the functionality of said RBS.

The functionality of the inferred promoter sequences and of the said presumed RBS having the ATG start codon in bringing genes without these signals to expression is demonstrated in the construction of plasmid pNZ280 in E.coli. This plasmid consists of plasmid pMC1403 (J.Bacteriol. 143 (1980) 971-980) in which the 495 bp promoter fragment has been introduced into the unique BamHI recognition site. The β-galactosidase (β-Gal) gene does not come to expression in pMC1403 because both the promoter and RBS of the β-Gal gene in pMC1403 are missing; the first seven amino terminal amino acids are also missing.

Expression of the β-Gal gene can only be brought about if the inserted fragment has promoter activity, an RBS with ATG start codon and a reading frame which is in phase with the eighth codon of the β-Gal segment.

Five µg of pMC1403 DNA were digested with 20 units of the restriction enzyme Bam HI, treated with phenol, concentrated and taken up in 50 µl of TE buffer. Purified 495 bp promoter fragment (for which the starting point was 5 µg of pNZ250 DNA as described earlier) was ligated with 5 µl of linearized pMC1403 DNA by means of T4 DNA ligase. Competent cells of E.coli strain MC1061 were transformed with the ligation mixture, and plated out on LB plates which contained 100 µg of carbenicillin and 40 µg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside per ml. In addition to a large number of colourless transformants, blue-coloured transformants also proved to be present; blue coloration indicates β-galactosidase activity - (Experiments in Molecular Genetics, Cold Spring Harbor 1972). In all the blue-coloured transformants investigated the 495 bp promoter fragment appeared to be inserted in th unique BamHI recognition site of pMC1403, while the plasmid DNA of th colourless transformants corresponded to the structure of pMC1403. Finally, the functionality of the 495 bp Bam HI promoter fragment in lactic acid bacteria was demonstrated by isolating said fragment as a 0.6 kb SalI fragment from pNZ221 (Figure 6) and inserting it

in the unique SalI recognition site which is upstream of the P-β-Gal gene in pNZ36 (Figure 7). The plasmid pNZ36 is derived from pNZ32 (see Example IV) and is constructed by removing the approximately 0.6 kb SalI-SstI fragment from pNZ32 and treating the residual fragment with DNA polymerase I (Klenow fragment) and T4 DNA polymerase (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor 1982), as a result of which a SalI recognition site is again reformed after circularization by means of T4 DNA ligase.

The promoter fragment is cloned in two orientations in said SalI recognition site: the promoter sequence in the same direction as the P-β-Gal gene resulting in pNZ367, and the promoter sequence in the opposite direction resulting in pNZ361. Both plasmids have been transformed into S.lactis and the expression of the P-β-Gal gene has been measured in both cases (Table 3). It emerges that high P-β-Gal activity is measured only if the promoter fragment is located in the correct orientation for the P-β-Gal gene, which indicates that said promoter fragment functions efficiently in lactic acid bacteria.

TABLE 3

P-β-Gal activity (nmol/min/mg of protein) in S.lactis MG1363

pNZ361	20
pNZ367	78

Legend

Figure 1:
Physical maps of the plasmids pGL112, pAT153, pNZ10 and pNZ12 showing the location of the antibiotic-resistance genes.

Figure 2:
Physical map of the S.lactis plasmid pSH71 and the nucleotide sequence of said plasmid.

Figure 3:
Physical map of the plasmids pSM76 and pNZ32 showing the location of the antibiotic-resistance genes and the P-β-Gal gene.

Figure 4:
Physical map of the plasmids pNZ22, pNZ12 and pNZ220. The recognition sites for the restriction enzymes EcoRI, XmaI, BamHI, SalI, PstI, HindIII, TaqI, MboI are indicated by the letters E, X, B, S, P, H, T and M respectively. MboI is an isoschizomer of Sau3A and XmaI of Sma I. The location of the antibiotic-resistance genes is indicated, the promoterless CAT gene of B.pumilis being shown by a broken line. The emboldened lines in pNZ12 and pNZ22 indicate the sections which are combined in pNZ220.

Figure 5:
Nucleotide sequence of the 495 bp Sau3A fragment of pNZ250 containing two promoter sequences (underlined), an RBS (dotted line) and an open reading frame starting with an ATG initiation codon th translation of which into amino acids is given.

Figure 6:
Physical map of the expression vector pNZ221 showing the location of the antibiotic-resistance genes; the promoterless CAT gene of B.pumilis is shown by a broken line.

Figure 7:
Physical map of the plasmid pNZ36 showing the location of the antibiotic-resistance genes and also the location and orientation of the P-β-Gal gene.

Claims

1. DNA fragment, characterized in that it contains (a) a DNA partial fragment which codes for a replicon activity in lactic acid bacteria and (b) at least on antibiotic-resistance gene.

2. DNA fragment according to claim 1, characterized in that it contains (a) a DNA partial fragment originating from lactic acid bacteria which cod s for a replicon activity in lactic acid bacteria and (b) at least one antibiotic-resistance gene.

3. DNA fragment according to claim 1 or 2, characterized in that it contains a kanamycin (Km)-resistance gene and/or a chloramphenicol (Cm)-resistance gene.

4. DNA fragment according to one or more of the claims 1 -3, characterized in that the DNA partial fragment 15which codes the replicon activity essentially has the DNA sequence 2 - 1680 as shown in Figure 2.

5. DNA fragment, characterized in that it has a DNA sequence which codes for a promoter activity in lactic acid bacteria.

6. DNA fragment according to claim 5, characterized in that the DNA fragment has essentially the DNA sequence as shown in Figure 5.

7. Method for isolating DNA fragments which code for replicon activity, characterized in that the replicon screening vector shown in Figure 1 is used in those micro-organisms in which at least one antibiotic-resistance gene is expressed, but in which the E.coli replicon is not functional.

8. Method for isolating DNA fragments which code for promoter activity, characterized in that a promoter screening vector as shown in Figure 4 is used in lactic acid bacteria.

9. Recombinant DNA plasmid, characterized in that it contains (a) a DNA fragment which codes for a replicon activity in lactic acid bacteria and (b) at least one antibiotic-resistance gene.

10. Recombinant DNA plasmid according to claim 9, characterized in that it contains (a) a DNA fragment originating from lactic acid bacteria which codes for a replicon activity in lactic acid bacteria and - (b) at least one antibiotic-resistance gene.

11. Recombinant DNA plasmid according to claim 9 or 10, characterized in that it contains a kanamycin (Km)-resistance gene and/or a chloramphenicol (Cm)-resistance gene.

12. Recombinant DNA plasmid according to one or more of the claims 9 -11, characterized in that the DNA fragment which codes for a replicon activity essentially has the DNA sequence 2 -16 80 as shown in Figure 2.

13. Recombinant DNA plasmid according to one or more of the claims 9 -12, characterized in that it also contains a DNA fragment which codes for a promoter activity and a ribosome binding site in lactic acid bacteria.

14. Recombinant DNA plasmid according to claim 13, characterized in that the DNA fragment which codes for the promoter activity and the ribosome binding site in lactic acid bacteria has essentially the DNA sequence as shown in Figure 5.

15. Recombinant DNA plasmid according to claim 13 or 14, characterized in that it also contains a gene which codes for a specific protein.

16. Recombinant DNA plasmid according to claim 15, characterized in that the gene is the phospho-β-galactosidase gene or a gene which codes for chymosin or another caseolytic enzyme or a precursor thereof.

17. Host organism containing one or more recombinant DNA plasmids, characterized in that the recombinant DNA plasmids correspond to the plasmids defined in claims 9 -16.

18. Host organism according to claim 17, characterized in that the host organism comprises lactic acid bacteria, S.aureus, B.subtilis or E.coli.

19. Host organism according to claim 18, characterized in that the host organism comprises lactic acid bacteria.

20. Host organism according to claim 19, characterized in that the host organism is S.lactis.

21. Method for the preparation of proteins by cultivating a microorganism containing one or more recombinant DNA plasmids, characterized in that a microorganism as defined in one or more of the claims 17 -20 is cultivated.

22. Protein obtained using the method according to claim 21.

fig-1

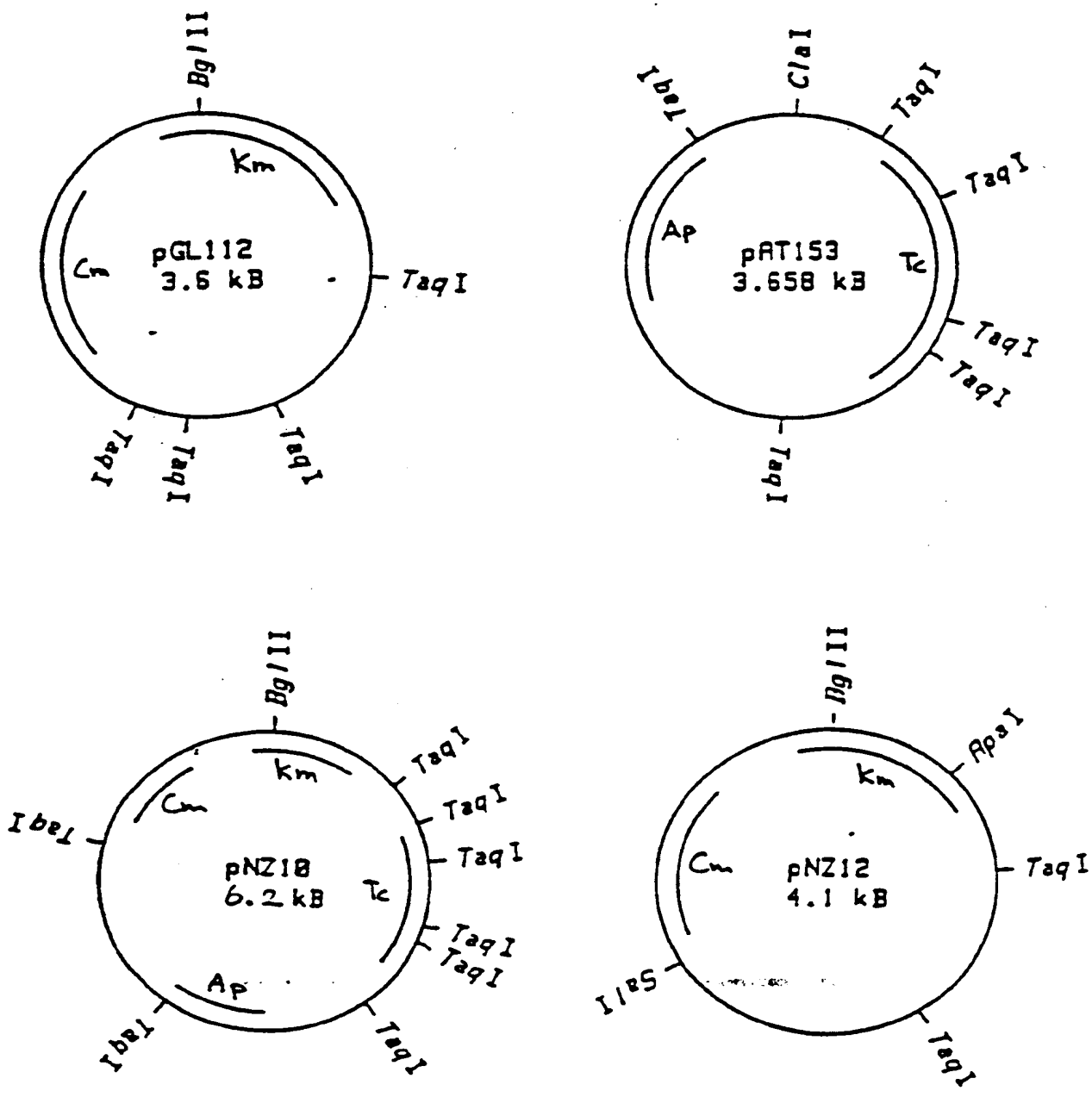

Fig-2 (PART 1)

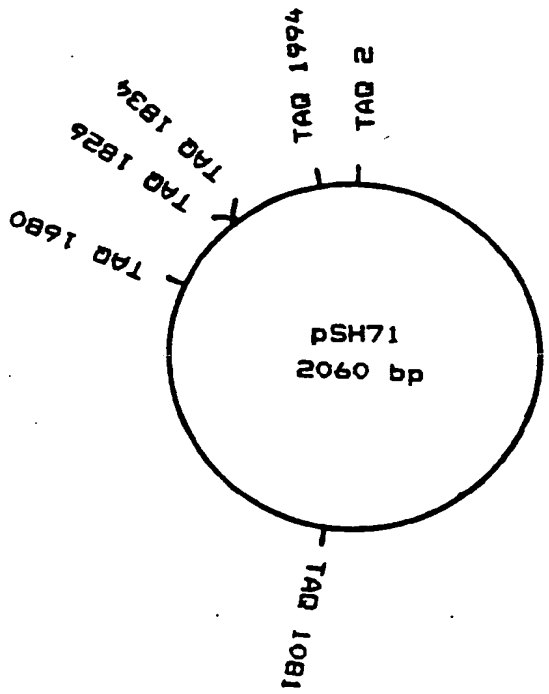

Fig-6

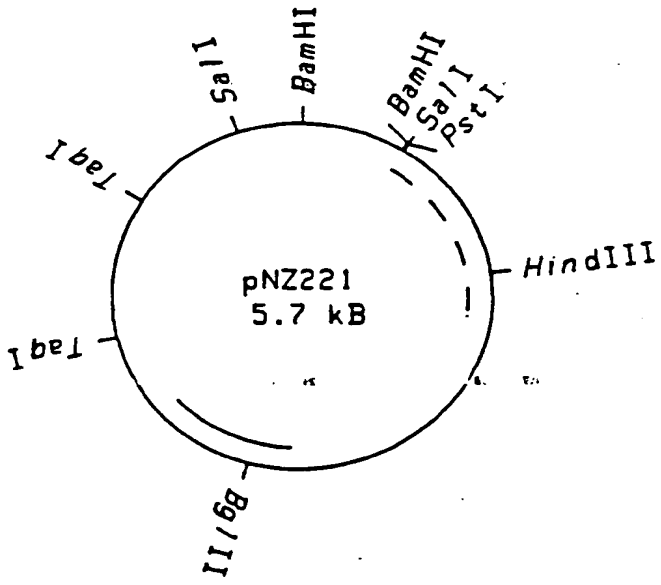

Fig-2 (PART 2)

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          10         20         30         40         50         60
           |          |          |          |          |          |
   1 TCGACTTTCG TCAGGGGGGC TTTTATTTAT TCAATAATCC CTCCTCTCAA TAAATCTATT
     AGCTGAAAGC AGTCCCCCCG AAAATAAATA AGTTATTAGG GAGGAGAGTT ATTTAGATAA

  61 GTTGTACTTA ATTCAACTTC CATTTCTCTG TATCTTTCAA TACGCTCTTT TAAGTCCTTA
     CAACATGAAT TAAGTTGAAG GTAAAGAGAC ATAGAAAGTT ATGCGAGAAA ATTCAGGAAT

 121 ATTTCTTTTT TTAATTCCTC ATTTTCAGCA AATAACTCTT TTTCTTTGTT TGTCATTTTA
     TAAAGAAAAA AATTAAGGAG TAAAAGTCGT TTATTGAGAA AAAGAAACAA ACAGTAAAAT

 181 TTTCCCCCGT TTCAGCATCA AGAACCTTTG CATAACTTGC TCTATATCCA CACTGATAAT
     AAAGGGGGCA AAGTCGTAGT TCTTGGAAAC GTATTGAACG AGATATAGGT GTGACTATTA

 241 TGCCCTCAAA CCATAATCTA AAGGCGCTAG AGTTTGTTGA AACAATATCT TTTACATCAT
     ACGGGAGTTT GGTATTAGAT TTCCGCGATC TCAAACAACT TTGTTATAGA AAATGTAGTA

 301 TCGTATTTAA AATTCCAAAC TCCGCTCCCC TAAGGCGAAT AAAAGCCATT AAATCTTTTG
     AGCATAAATT TTAAGGTTTG AGGCGAGGGG ATTCCGCTTA TTTTCGGTAA TTTAGAAAAC

 361 TATTTACCAA ATTATAGTCA TCCACTATAT CTAAAAGTAA ATTCTTCAAT TCTCTTTTTT
     ATAAATGGTT TAATATCAGT AGGTGATATA GATTTTCATT TAAGAAGTTA AGAGAAAAAA

 421 GGCTTTCATC AAGTGTTATA TAGCGGTCAA TATCAAAATC ATTAATGTTC AAAATATCTT
     CCGAAAGTAG TTCACAATAT ATCGCCAGTT ATAGTTTTAG TAATTACAAG TTTTATAGAA

 481 TTTTGTCGTA TATATGTTTA TTCTTAGCAA TAGCGTCCTT TGATTCATGA GTCAAATATT
     AAAACAGCAT ATATACAAAT AAGAATCGTT ATCGCAGGAA ACTAAGTACT CAGTTTATAA

 541 CATATGAACC TTTGATATAA TCAAGTATCT CAACATGAGC AACTGAACTA TTCCCCAATT
     GTATACTTGG AAACTATATT AGTTCATAGA GTTGTACTCG TTGACTTGAT AAGGGGTTAA

 601 TTCGCTTAAT CTTGTTCCTA ACGCTTTCTA TTGTTACAGG ATTTCGTGCA ATATATATAA
     AAGCGAATTA GAACAAGGAT TGCGAAAGAT AACAATGTCC TAAAGCACGT TATATATATT

 661 CGTGATAGTG TGGTTTTTTA TAGTGCTTTC CATTTCGTAT AACATCACTA CTATTCCATG
     GCACTATCAC ACCAAAAAAT ATCACGAAAG GTAAAGCATA TTGTAGTGAT GATAAGGTAC

 721 TATCTTTATC TTTTTTTTCG TCCATATCGT GTAAAGGACT GACAGCCATA GATACGCCCA
     ATAGAAATAG AAAAAAAAGC AGGTATAGCA CATTTCCTGA CTGTCGGTAT CTATGCGGGT

 781 AACTCTCTAA TTTTTCTTTC CAATCATTAG GAATTGAGTC AGGATATAAT AAAAATCCAA
     TTGAGAGATT AAAAAGAAAG GTTAGTAATC CTTAACTCAG TCCTATATTA TTTTTAGGTT

 841 AGTTTCTAGC TTTAGTATTT AATAGCCATG ATATATTACC TTATCAAAAA CAAGTAGCGA
     TTAAAGATCG AAATCATAAA TTATCGGTAC TATATAATGG AATAGTTTTT GTTCATCGCT

 901 AAACTCGTAT CCTTCTAAAA ACGCGAGCTT TCGCTTATTT TTTTTATTCT GATTCCTTTC
     TTTGAGCATA GGAAGATTTT TGCGCTCGAA AGCGAATAAA AAAAATAAGA CTAAGGAAAG

 961 TTGCATATTC TTCTATAGCT AACGCCGCAA CCGCAGATTT TGAAAAACCT TTTTGTTTCG
     AACGTATAAG AAGATATCGA TTGCGGCGTT GGCGTCTAAA ACTTTTTGGA AAAACAAAGC

1021 CCATATCTGT TAATTTTTTA TCTTGCTCTT TTGTCAGAGA AATCATAACT CTTTTTTTCG
     GGTATAGACA ATTAAAAAAT AGAACGAGAA AACAGTCTCT TTAGTATTGA GAAAAAAAGC

1081 ATTCTGAAAT CACCATTTAA AAAACTCCAA TCAAATAATT TTATAAAATT AGTGTATCAC
     TAAGACTTTA GTGGTAAATT TTTTGAGGTT AGTTTATTAA AATATTTTAA TCACATAGTG

1141 TTTGTAATCA TAAAAACAAC AATAAAGCTA CTTAAATATA GATTTATAAA AAACGTTGGC
     AAACATTAGT ATTTTTGTTG TTATTTCGAT GAATTTATAT CTAAATATTT TTTGCAACCG
```

Fig-2 (PART 3)

```
1201 GAAAACGTTG GCGATTCGTT GGCGATTGAA AAACCCCTCA AACCCTTGAG CCAGTTGGGA
     CTTTTGCAAC CGCTAAGCAA CCGCTAACTT TTTGGGGAGT TTGGGAACTC GGTCAACCCT

1261 TAGAGCGTTT TTGGCACAAA AATTGGCACT CGGCACTTAA TGGGGGGTCG TAGTACGGAA
     ATCTCGCAAA AACCGTGTTT TTAACCGTGA GCCGTGAATT ACCCCCCAGC ATCATGCCTT

1321 GCAAAATTCG CTTCCTTTCC CCCCATTTTT TTCCAAATTC CAAATTTTTT TCAAAAATTT
     CGTTTTAAGC GAAGGAAAGG GGGGTAAAAA AAGGTTTAAG GTTTAAAAAA AGTTTTTAAA

1381 TTCCAGCGCT ACCGCTCGGC AAAATTGCAA GCAATTTTTA AAATCAAACC CATGAGGGAA
     AAGGTCGCGA TGGCGAGCCG TTTTAACGTT CGTTAAAAAT TTTAGTTTGG GTACTCCCTT

1441 TTTCATTCCC TCAAACTCCC TTGAGCCTCC TCCAACCGAA ATAGAAGGAC GCTGCGCTTA
     AAAGTAAGGG AGTTTGAGGG AACTCGGAGG AGGTTGGCTT TATCTTCCTG CGACGCGAAT

1501 TTATTTCATT CAGTCATCGG CTTTCATAAT CTAACAGACA ACATCTTCGC TGCAAAGCAC
     AATAAAGTAA GTCAGTAGCC GAAAGTATTA GATTGTCTGT TGTAGAAGCG ACGTTTCGTG

1561 GCTACGCTCA AGGGCTTTTA CGCTACGATA ACGCCTGTTT TAACGATTAT GCCGATAACT
     CGATGCGAGT TCCCGAAAAT GCGATGCTAT TGCGGACAAA ATTGCTAATA CGGCTATTGA

1621 AAACGAAATA AACGCTAAAA CGTCTCAGAA ACGATTTTGA GACGTTTTAA TAAAAAATCG
     TTTGCTTTAT TTGCGATTTT GCAGAGTCTT TGCTAAAACT CTGCAAAATT ATTTTTTAGC

1681 ATAAATTATA TTGGCAAATT ATAAAAATGC CGTAGAACGA AAAATAGAGC CCTTAAAAAG
     TATTTAATAT AACCGTTTAA TATTTTTACG GCATCTTGCT TTTTATCTCG GGAATTTTTC

1741 CAAATAAACT ACGATAGCAA CAAAAAACAA AACCAAAACC CAAAAGATGA AAAATAAATA
     GTTTATTTGA TGCTATCGTT GTTTTTTGTT TTGGTTTTGG GTTTTCTACT TTTTATTTAT

1801 CAACAGCAAA AATAAATCAT AGTTCGATTT TTCGAACAAT GCGCACTTAC ACACCACTCC
     GTTGTCGTTT TTATTTAGTA TCAAGCTAAA AAGCTTGTTA CGCGTGAATG TGTGGTGAGG

1861 AAAATTGGGT GGTTTTTGTG CTTAAAAAAA TGTATCAGAA GTCGGCTAGC CGACAACAAA
     TTTTAACCCA CCAAAAACAC GAATTTTTTT ACATAGTCTT CAGCCGATCG GCTGTTGTTT

1921 AAAAGCGCTA ATAAATTAGC GCCATAAATA AAATATAAAT TAATCACTTT TTCTTTTTTT
     TTTTCGCGAT TATTTAATCG CGGTATTTAT TTTATATTTA ATTAGTGAAA AAGAAAAAAA

1981 GCCAAACAGC ATCGATCATT TTGTTTATTG CAATCGTATT GCTATTAATC GCAACATCAA
     CGGTTTGTCG TAGCTAGTAA AACAAATAAC GTTAGCATAA CGATAATTAG CGTTGTAGTT

2041 ACCAAAATAA AAGCCCCCT
     TGGTTTTATT TTCGGGGGA
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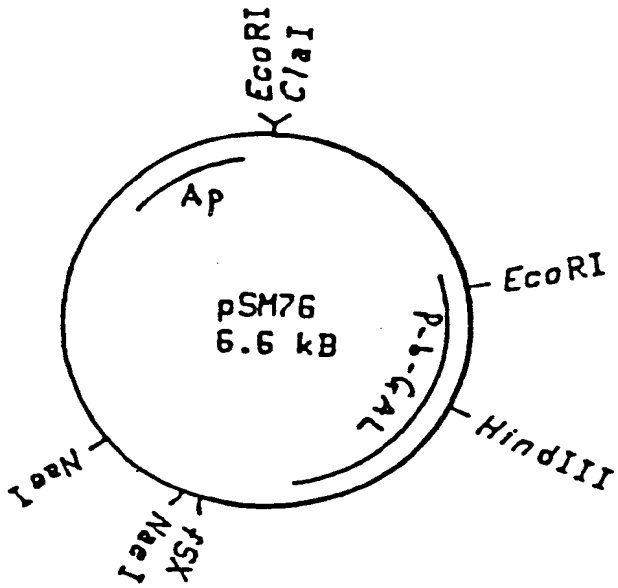
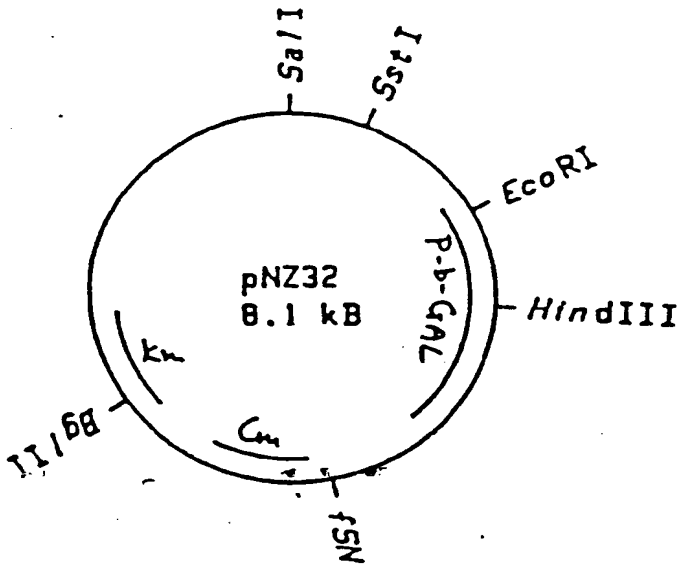
Total number of bases is: 2059.
DNA sequence composition: 672 A; 424 C; 263 G; [illegible] T;

Sequence coded: NFSH71P.

fig-3

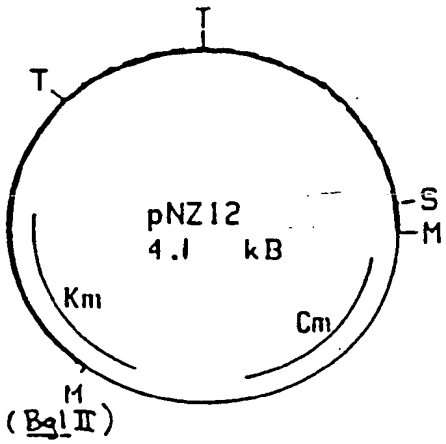


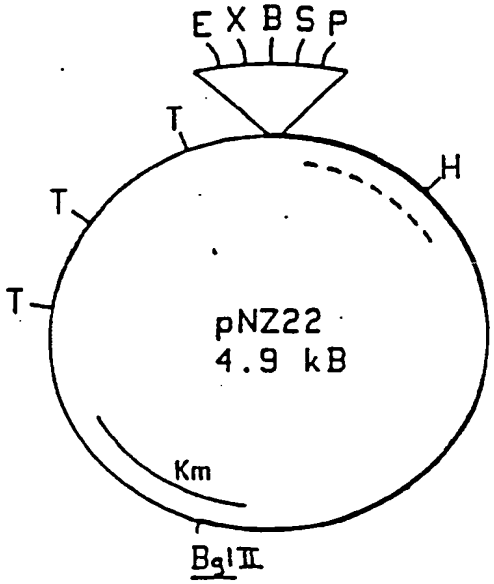
fSX = SalI - XhoI FUSION



fSN = SalI-NaeI FUSION

Fig-4





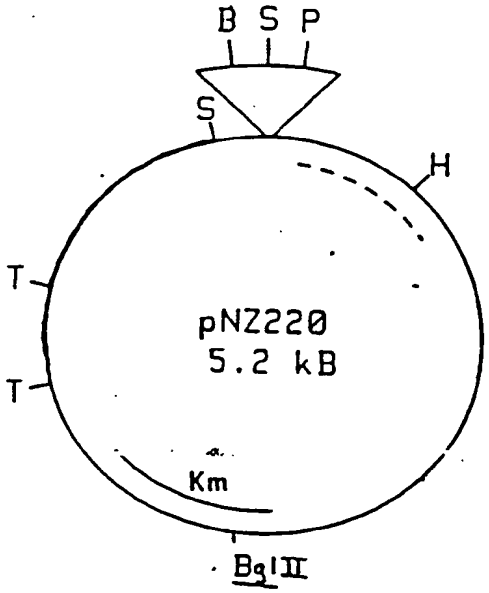
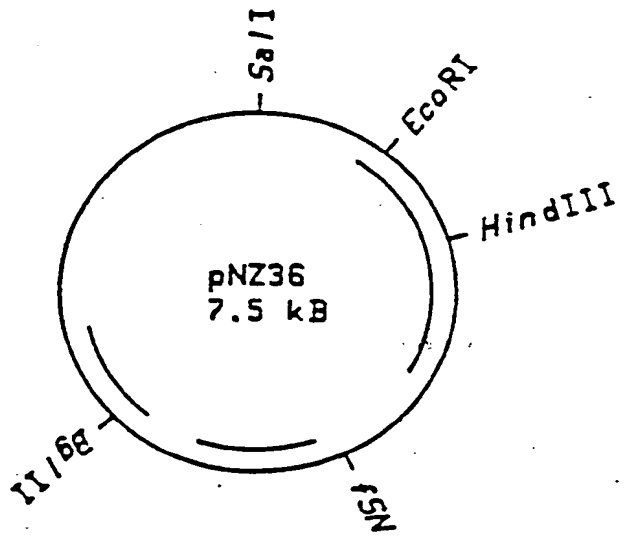


Fig-5

```
        10         20         30         40         50         60         70
        :          :          :          :          :          :          :
GGATCCAACTTGAAAAGGAACTGACTTATAAACTGATTTCAACAAACCGCAGACGAGTAGTTAAGGGTAATT

      80         90        100        110        120        130        140
      :          :          :          :          :          :          :
TATATTATGAACGTATAATCGTAGATAACCAGCTATTTAAGTTTGCAACTTTGAAAGATTTAATTGACTTGT

   150        160  HaeIII 170        180        190        200        210
    :          :          :          :          :          :          :
ATCACGAAAATATTAATGGGCCTATTTTTAGACAGAATCAGCTTCTTGTTAAAAIGGGAGAGCAACCAATCG

  220        230        240        250        260        270        280
   :          :          :          :          :          :          :
AGGGTGGAGATATTTAACATAGCTAACCTTAACGCAGTTGCTGTTAAAAACCTAAGTGACCTACAAGGCAGT

290        300        310        320        330        340        350        360
 :          :          :          :          :          :          :          :
AGAAAGGACGTAACAAGCACAGATGAAACTAATAACCAATAGTGCTGAAATTAAAGTAACTGAAAATGAGGA

                                                  METLysLeuIleThrAsnSerAlaGluIleLysValThrGluAsnGluAsp

       370        380        390        400        410        420        430
        :          :          :          :          :          :          :
CGGTTCTAAGTCGTTCCAAGGAATTGGTTCAGAAGTTGGTGTAGACAATCTTAACGGTATTGTCTTGACACC

 GlySerLysSerPheGlnGlyIleGlySerGluValGlyValAspAsnLeuAsnGlyIleValLeuThrPro

      440        450        460        470        480        490
       :          :          :          :          :          :
TAACTGCATTGAGTTTGCTAGAGAACGATATCCATTGCTATATATGAACACGGAGCTGGATCC

 AsnCysIleGluPheAlaArgGluArgTyrProLeuLeuTyrMETAsnThrGluLeuAsp
```

Fig-7



European Patent Office

EUROPEAN SEARCH REPORT

Application number

EP 86 20 2061

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
D,X	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 48, no. 4, October 1984, pages 726-731, American Society for Microbiology, US; J. KOK et al.: "Construction of plasmid cloning vectors for lactic Streptococci which also replicate in Bacillus subtilis and Escherichia coli" * Whole document * ---	1-3,5, 9-11, 13,15-22	C 12 N 15/00 C 12 N 1/20 C 12 P 21/02 (C 12 N 1/20 C 12 R 1:07 C 12 R 1:19 C 12 R 1:46)
X	WO-A-8 503 945 (UNILEVER N.V.) * Whole document * ---	1-3,5, 9-11, 13,15-22	
X	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 50, no. 2, August 1985, pages 540-542, American Society for Microbiology, US; J.M.B.M. VAN DER VOSSEN et al.: "Construction of cloning, promoter-screening, and terminator-screening shuttle vectors for Bacillus-subtilis and Streptococcus lactis" * Whole document *	1-3,5, 9-11, 13,17-22	TECHNICAL FIELDS SEARCHED (Int Cl 4) C 12 N C 12 P
A	Idem --- -/-	8	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	12-03-1987	DESCAMPS J.A.

CATEGORY OF CITED DOCUMENTS

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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl 4)
X	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 50, no. 1, July 1985, pages 94-101, American Society for Microbiology, US; J. KOK et al.: "Cloning and expression of a Streptococcus cremoris proteinase in Bacillus subtilis and Streptococcus lactis" * Figure 3; page 97, column 1, paragraph 2 - page 99, column 2, paragraph 1 *	1-3,5, 9-11, 13,15-22	
X	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 49, no. 1, January 1985, pages 115-119, American Society for Microbiology; MY LIEN DAO et al.: "Streptococcus-Escherichia coli shuttle vector pSA3 and its use in the cloning of Streptococcal genes" * Whole document *	1-3,5, 9-11, 13,15, 17-19, 21,22	
P,X	NETH. MILK DAIRY J., vol. 40, no. 2/3, 1986, pages 141-154; W.M. DE VOS: "Gene cloning in lactic Streptococci" * Whole document *	1-3,5, 8-11, 13,15-22	

TECHNICAL FIELDS SEARCHED (Int. Cl 4)

-/-

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Application number

EP 86 20 2061

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 48, no. 2, August 1984, pages 252-259, American Society for Microbiology; J.K. KONDO et al.: "Plasmid transformation of Streptococcus lactis protoplasts: optimization and use in molecular cloning" -----		

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

The present search report has been drawn up for all claims

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